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Re-expression of *SPR1* in Breast Cancer Cells by Phorbol 12-Myristate 13-Acetate (PMA) or UV Irradiation Is Mediated by the AP-1 Binding Site in the *SPR1* Promoter

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Abstract

Background: Invasive tumor cells are characterized by multiple phenotypic changes as a result of the large number of cDNAs being differentially expressed in tumor cells compared to normal progenitors. Expression genetics focuses on changes at the RNA level with the aim of identifying functionally important genes whose aberrant expression in cancer cells is regulated at the level of transcription. These genes were named class II genes and are distinguished from class I genes, which are characterized by genomic mutations, deletions, or other alterations. Reversal of the tumor cell phenotype accompanying normalization of the expression of such genes may be exploited therapeutically if gene expression can be specifically modulated by drugs or other treatments. Considering that genes are coordinately regulated in complex networks, it is likely that the expression of multiple genes can be simultaneously modulated in tumor cells by drugs acting on the signal transduction pathway that regulates their expression. The *SPR1* gene is associated with differentiation and its expression is down-regulated or inactivated in malignant cells. Analysis of the *SPR1* promoter showed that down-regulation of *SPR1* expression in breast tumor cells occurs at the level of transcription. *SPR1* presents an example of class II genes, since its expression was up-regulated in tumor cells by phorbol 12-myristate 13-acetate (PMA) or by ultraviolet (UV) irradiation.

Materials and Methods: The *SPR1* gene was identified by differential display on the basis of its reduced or absent expression in human breast tumor cell lines compared to normal mammary epithelial cell strains. Differential expression was confirmed by Northern blot analysis employing multiple normal and tumor cell lines. The promoter region −619 to +15 of the *SPR1* gene was sequenced and analyzed by CAT assays, deletion analysis, and mutagenesis. Up-regulation of *SPR1* expression by PMA and UV irradiation was monitored by Northern analysis and analyzed by CAT assays.

Results: The mechanism of down-regulation of *SPR1* expression in breast tumor cells was investigated. It was found that the −619 to +15 upstream promoter region is sufficient for *SPR1* expression in normal breast cells, but it is transcriptionally silent in most breast tumor cell lines. By deletion analysis and mutagenesis, two upstream *cis*-acting promoter elements were identified. Our data indicate that the AP-1 element located between −139 and −133 acts as a major enhancer of *SPR1* transcription only in normal mammary epithelial cells but not in corresponding tumor cells, whereas the sequences flanking the AP-1 site do not affect its promoter enhancing activity. In addition, a transcriptional repressor was identified that binds unknown factor(s) and is active in both normal and tumor breast cells. Inhibitor function was mapped to a 35-bp element located from −178 to −139 upstream of the human *SPR1* mRNA start site. The expression of *SPR1* could be induced in the 21MT-2 metastatic breast tumor cell line by PMA treatment or by short UV irradiation via a transcriptional mechanism. AP-1 is the *cis* element mediating the transcriptional activation of *SPR1* by PMA, which induces the expression of AP-1 factors in 21MT-2 cells. Mutation of the AP-1 site abolishes the induction of *SPR1* expression by PMA.

Conclusions: Our results demonstrate that loss of *SPR1* expression in breast tumor cells results from impaired transactivation through the AP-1 site in the *SPR1* promoter, as well as from the presence of a negative regulatory element active in both normal and tumor cells. Furthermore, our results provide a basis for therapeutic manipulation of down-regulated genes, such as *SPR1*, in human cancers.

Introduction

The novel concept of RNA genetics in cancer underscores the importance of changes in the expression of critical genes in understanding and treating cancer (1). Instead of studying mutations or other gene alterations at the genome level, expression genetics focuses on changes at the RNA level. We have previously applied methods of differential expression cloning, such as subtractive hybridization (2) and differential display (DD) (3,4), to the identification of genes whose expression is up- or down-regulated in breast cancers. Several novel genes were identified and their putative functional role(s) in regulating the growth and/or dissemination of mammary tumors was investigated (4–7). Differential display allows the quantitative evaluation of cellular messenger RNA patterns. Comparison of well-matched cell lines showed remarkable similarity in expression profiles of normal and tumor breast cells, while the percentage of differentially expressed messages was only 0.5–1%. Our results indicated that the number of complementary DNAs (cDNAs) differentially expressed between normal and metastatic breast cancer cells lies in the range of 500 to 1000 (1,5). Similar data were obtained by others who analyzed differential gene expression in human colon cancer by means of serial analysis of gene expression (SAGE) (8). The large number of differentially expressed cDNAs is consistent with the multiple phenotypic changes characteristic of metastatic tumor cells. Functionally important genes, whose aberrant expression in tumor cells is regulated at the level of transcription, can possibly be targeted for therapy if their normal expression can be induced by drugs. Therefore, it is essential to delineate the mechanism(s) underlying the aberrant regulation of transcription of cancer-related genes. In this report, the small proline-rich protein gene (*SPR1*) was identified by DD on the basis of its reduced or absent expression in human breast tumor cell lines compared to normal counterparts. Evidence is presented that down-regulation of *SPR1* in breast tumor cells occurs by a transcriptional mechanism.

Small proline-rich (*SPR*) proteins are characterized by an unusually high content in proline residues and were originally identified in cultured keratinocytes as ultraviolet (UV)-inducible genes (9,10). *SPR* proteins are rich in cysteine and glutamine residues and contain repeating elements unique to the SPR family. Similarly, the *SPRR* genes constitute part of the human epidermal differentiation complex and their expression is strictly linked to keratinocyte terminal differentiation (11). Squamous carcinoma cell lines, on the other hand, show significantly lower levels of *SPRR* expression than normal human keratinocytes (12). The *SPR1* protein is a component of the cross-linked envelope that forms during the squamous differentiation process and was suggested as a marker associated with squamous cell differentiation, including the airway epithelium (13,14). Although human normal bronchial epithelial cells express high levels of *SPR1*, expression of *SPR1* measured by quantitative reverse transcription–polymerase chain reaction (RT-PCR) was inactivated in malignant human bronchial epithelial cells. Therefore, loss of *SPR1* gene expression likely contributes to malignant transformation by disrupting mechanisms for terminal squamous differentiation (15). The differential expression of *SPR1* in breast normal and tumor cell lines described in this report indicates that the *SPR1* protein could also play a significant role in the mammary epithelium. Therefore, the molecular mechanism(s) underlying the differential transcriptional regulation of *SPR1* was examined. Analysis of the *SPR1* promoter showed that in normal human breast cells *SPR1* transcription is regulated by the coordinated action of two major *cis* elements: the mammalian activating protein-1 (AP-1) site located at −139 to −133 being the positive regulator and a negative regulator of *SPR1* transcription located at −178 to −139. The *SPR1* gene promoter was characterized in a previous study (16,17), which indicated the presence of both phorbol ester and cAMP-responsive elements. We have demonstrated that loss of *SPR1* expression in breast tumor cells results from the absence of transactivation through the AP-1 binding site in combination with negative regulation through the inhibitory element, and can be restored in 21MT-2 tumor cells by phorbol 12-myristate 13-acetate (PMA) treatment and by UV irradiation.

This manuscript is written in memory of our mentor and friend, Professor Ruth Sager.

The sequence reported in this manuscript appears in the GenBank™/EMBL database with the accession number AF079516.

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Materials and Methods

Cell Strains, Cell Lines, and Growth Media

Normal human mammary epithelial cell strains (81N, 76N, and 70N) were derived from reduction mammoplasties. Primary (21NT, 21PT) and metastatic (21MT-1, 21MT-2) tumor cell lines were established from a single patient (18). Metastatic mammary epithelial tumor cell lines MCF7, BT474, BT549, T47D, ZR-75-1, MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-435, and MDA-MB-436 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell cultures were routinely grown in DFCl-1 medium (19). Cultures were grown in P100 dishes and harvested at about 75% confluence for RNA isolation and near confluence for DNA isolation. *Escherichia coli* K12 bacteria strain *XL-1* Blue was obtained from Stratagene (La Jolla, CA). Tissue culture medium components were purchased from Life Technologies (Bethesda, MD) or Hyclone (Logan, UT).

Differential Display

Total cellular RNAs (50 μg) from exponentially growing cell cultures were treated with DNase I in the presence of RNasin ribonuclease inhibitor to remove any residual DNA contamination as described elsewhere (4). RNAs were extracted with phenol/chloroform, precipitated with ethanol, and redissolved in DEPC-treated water. Subsequently, the RNAs were reverse transcribed using a 3′-anchored primer $T_{12}MG$ (5′-TTT-TTTTTTTTTMG-3′) (Operon Technologies, Alameda, CA). The resultant cDNAs were amplified by PCR, in the presence of [^{35}S]dATP, using $T_{12}MG$ as the 3′-end primer and OPA1 (5′-CAG-GCCCTTC-3′), an arbitrary 10-mer primer, as the 5′-end primer and resolved side-by-side on a 6% acrylamide/urea sequencing gel. The partial cDNA bands were localized by autoradiography and eluted as described elsewhere (4). A differentially displayed cDNA of ~0.16 kilobases (kb) was recovered from the dried gel, purified by a Millipore Ultrafree MC unit, reamplified by PCR, ^{32}P-labeled by the oligolabeling method (20), and used as a probe for hybridization of Northern blots. The partial cDNA obtained from DD was subcloned into the PCRII vector (Invitrogen), and a corresponding full-length cDNA clone was isolated and sequenced on both strands with T7 and SP6 primers. A cDNA library from 76N cells constructed in λZap II (Stratagene, San Diego, CA) was screened using the cloned PCR product as a probe and several full-length cDNA clones were isolated. The differential expression of *SPR1* was confirmed by Northern hybridizations of the same RNA samples used for DD, as well as RNAs from a number of normal and tumor cell lines, using a full-length probe. Full-length cDNA clones were sequenced on both strands. Sequencing was performed with an ABI automated sequencer, Model 373A, in the Molecular Biology Core Facility of the Dana-Farber Cancer Institute. The basic local alignment search tool (BLAST) algorithm was used for nucleic acid sequence comparisons (21).

Northern and Southern Blot Analysis

Total cellular RNA was isolated from cells cultured to approximately 75% confluence, purified by standard guanidinium isothiocyanate and cesium chloride centrifugation, and analyzed as described (4). For Northern blotting, 20 μg of total RNA was resolved by electrophoresis on 1% agarose–1.7 M formaldehyde gels, transferred to a Zetaprobe membrane (BioRad, Richmond, CA) in 20×SSC solution (20×SSC, 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0), and baked for 1 hr at 80°C. Hybridizations were performed in formamide at 37°C overnight. The blot was washed at 65°C for 1 hr in 2×SSC containing 0.1% SDS. As an internal loading control, 36B4 was used, a gene encoding a ribosomal protein whose expression is not affected by growth conditions or estrogen receptor expression (22). For Southern blotting, 10 μg of genomic DNA was digested with *Eco*RI overnight at 37°C, fractionated on a 1.5% agarose gel, transferred to nylon membrane, and hybridized as above. Genomic DNA was isolated and hybridized by standard methods (23). Densitometric scans of autoradiographs were obtained with the BioRad GS-700 imaging densitometer using Molecular Analyst software.

Isolation of Genomic Clones: Promoter Cloning and Sequencing

Genomic clones for the *SPR1* gene were isolated from a human white female leukocyte *Mbo*I partial library in λEMBL 3. Approximately 1×10^6 plaques were screened with a ^{32}P-labeled *SPR1* full-length cDNA probe. Single hybridizing plaques were isolated after three rounds of screening picked into SM buffer (23) and amplified by PCR (95°C, 1 min; 55°C, 1 min; 72°C, 1 min; 35 cycles) with −622 and CAP primers (see oligonucleotide section below). Subsequently,

the 0.63 kb PCR product was cloned into the PCRII vector (Invitrogen). Plasmid DNA was prepared and digested with *Hin*dIII and *Sal*I; the 0.63 kb promoter fragment was purified on Qiaex (Qiagen, Chatsworth, CA) and cloned into the promoterless chloramphenicol acetyltransferase (CAT) vector pKT (24). The promoter containing plasmid was sequenced at the Dana-Farber Cancer Institute Core Facility with an ABI 373A automated DNA sequencer (Applied Biosystmes, Foster City, CA). Genomic DNA from the 70N normal cell strain was similarly amplified by PCR with −622 and CAP primers, cloned into the PCRII vector, and sequenced as above.

Synthetic Oligonucleotides

For PCR amplification of the SPR1 promoter fragment the following oligonucleotides were used: −622-primer (5′-GGTCGACCAGAGTTCACTGCTCCGA-3′) and CAP primer (5′-CAAGCTTAGAACTGGTGGTAGTGTTCCC-3′) (modified from primer sequences used by R. Wu, personal communication). For construction of the pKT/*SPR1* promoter 5′ deletion mutants, the antisense CAP primer (5′-AAGCTTAAGCTTAGAACTGGTGGTAGTGTTCCC-3′) was used for PCR in combination with each of the following sense oligonucleotides: for p-224 (5′-GTCGACGTCGACCCTGGAGCAAAGGGTGTTCAG-3′); for p-133 (5′-GTCGACGTCGACAGGTGGGTGAGGGAAGAGGGG-3′). The following oligonucleotides were used for the preparation of CAT constructs: *SPR1* AP-1 WT sense (5′-TCGACCAAAAGT**TGAGTCA**ACAGGTGT-3′) and antisense (5′-CTAGACACCTGT**TGACTCA**ACTTTTGG-3′); AP-1 mut1 with two changes in the AP-1 site, sense (5′-TCGACCAAAAGT**GGAGTCC**ACCGGTGT-3′) and antisense (5′-CTAGACACCTGT**GGACTCC**ACTTTTGG-3′); AP-1 mut2 with seven changes in the AP-1 site: sense (5′-TCGACCAAAAGT**GCTCGAG**ACAGGTGT-3′) and antisense (5′-CTAGACACCTGT**CTCGAGC**ACTTTTGG-3′); consensus AP-1 WT1-1 (Promega): sense (5′-TCGACCGCTTGA**TGAGTCA**GCCGGAAT-3′) and antisense (5′-CTAGATTCCGGC**TGACTCA**TCAAGCGG-3′). Sense AP-1 oligos contained *Sal*I cloning sites (TCGAC) and antisense AP-1 oligos contained *Xba*I (CTAGA) cloning sites. Positions where mutations were introduced are underlined. For the construction of pBLCAT2/AP-1 INH, oligos containing the SPR1 inhibitor region (−178 to −139) were used: sense (5′-AGCTTCC**TTGTCAGACAGCAAGTGCCACAAGTTTCATCACAAAAGTG**-3′) and antisense (5′-TCGA**CACTTTTGTGATGAAACTTGTGGCACTTGCTGTCTGACAA**GGA-3′). The inhibitor oligos contained *Hin*dIII (AGCTT) and *Sal*I (TCGAC) cloning sites, respectively. The final pBLCAT2/AP-1 INH plasmid contained the inhibitor upstream of the AP-1 sequence ligated upstream of the thymidine kinase (TK) promoter. The presence of AP-1 and the inhibitor was confirmed by sequencing. Oligos were synthesized by Amitof (Boston, MA).

CAT and β-galactosidase (β-Gal) Constructs

The pBLCAT2 and pBLCAT3 CAT expression plasmids were used to test the activity of the *SPR1* promoter. The leukocyte promoter fragment from −619 to +15 was subcloned into the pKT promoterless CAT vector (24) to generate the p-619 construct. A nested series of progressive 5′-deletions were generated from p-619 using either restriction enzyme digestion, *Exo*III/S1 nuclease treatment, or PCR. P-619 was digested with restriction enzyme, blunt ended with T4 polymerase, and cut with *Hin*dIII (at +15), and the promoter fragment with the appropriate size was purified from the agarose gel on Qiaex and cloned into the pKT vector previously digested with *Sma*I and *Hin*dIII. The following deletions were made in this manner: p-477 (*Acc*I), p-297 (*Alw*NI), p-178 and p-161 (*Drd*I), and p-58 (*Bam*HI). The p-139 deletion was generated by digestion of p-619 with *Xba*I and *Sst*I followed by *Exo*III treatment, S1 nuclease treatment (Promega Biotech, Madison, WI), blunt ending with Klenow, and ligation (Erase-a-base system, Promega). The p-224 and p-133 deletion mutants were generated by PCR, at 95°C for 1 min, 55° or 60°C for 1 min, 72°C for 1 min for 30 cycles, with primers shown in the oligonucleotide section. The 5′ endpoints of all constructs were verified by sequencing with the M13R primer. The pBLCAT2 constructs were prepared by annealing the appropriate AP-1 oligonucleotides as described above and subsequent cloning into pBLCAT2 digested with *Sal*I and *Xba*I, so that the AP-1 sequences were upstream of the TK promoter. The pBLCAT2/INH AP-1 construct was prepared by annealing the inhibitor oligonucleotides and cloning into pBLCAT2 AP-1wt previously digested with *Hin*dIII and *Sal*I, so that the inhibitor sequence is just upstream of AP-1. All constructs were sequenced to confirm the expected structure. The pCMVβ-Gal contained the mouse cytomegalovirus (CMV) promoter up-

stream of the β-Gal gene and was constructed previously (25).

DNA Transfection and CAT Assays

Plasmids were prepared by alkaline lysis and column purified (Qiagen). About 1×10^6 cells were plated per P100 dish and grown to about 75% confluence. According to the method of Graham and van der Eb (26), 70N or 21MT-2 cells were transiently transfected for 4 hr with 20 μg of each *SPR1*/CAT reporter plasmids; 1 μg of a CMV-driven β-Gal expression vector (pCMV-CAT) was cotransfected as an internal control for transfection efficiency. The calcium phosphate co-precipitate was then thoroughly washed off, fresh medium [Eagle's Minimal Essential Medium (α-MEM)/0.5% fetal calf serum (FCS)] was added, and the plates were incubated at 37°C. Transient transfections of reporter plasmids into MDA-MB-231 and ZR-75-1 cell lines were carried out by the lipofectin method (Gibco, BRL, Gaithersburg, MD) or modified DEAE-Dextran (Promega, Madison, WI). Forty-eight hours after transfection, the cells were washed with phosphate-buffered saline (PBS) and harvested by scraping directly into 350 μl of 0.25 M Tris · HCl, pH 7.8, 15% glycerol. Extracts were prepared by three cycles of freezing in dry ice–ethanol and thawing at 37°C. Protein concentration was determined by the Bradford assay (BioRad). The β-Gal activity was determined by using 3 mg/ml chlorophenol red-β-D-galactopyranoside as substrate and reading the absorbance at 574 nm as described previously (27). CAT assays were performed on amounts of extracts giving equal β-Gal activity for the uninduced controls, in 250 μl final volume containing 0.8 mM acetyl CoA and 0.2 μCi of [Dichloroacetyl-1,2-^{14}C]-chloramphenicol (DuPont/New England Nuclear, Boston, MA) by the method of Gorman et al. (28). Twenty units of extracts (calculated by β-Gal activity) were routinely used for each CAT assay, while only 2 units of extract were used for transfection with the pCMVCAT-positive control because of its high activity. Quantitation of acetylated CoA and nonacetylated chloramphenicol was performed by excising the appropriate regions of the silica gel thin-layer chromatography (TLC) plate and counting radioactivity in BioFluor (DuPont, Wilmington, DE).

UV Treatment of Cells

The 21MT-2 cells were cultured to about 70% confluence. Before irradiation cells were washed several times with PBS, which was then removed, and cells were irradiated with short-wavelength UV light at the following doses: 0, 8, 17, 25, 33, and 42 J/M^2. Dose rates were determined with a UVX radiometer (29). After irradiation fresh growth medium was added and cells were incubated at 37°C for 12 or 24 hr before they were harvested for isolation of total cellular RNA. For CAT assays, 21MT-2 cells were transfected with reporter plasmids and cultured for 24 hr before UV irradiation. After UV irradiation, fresh growth medium was added, and cells were incubated at 37°C for 9, 24, or 32 hr prior to CAT assays.

PMA Treatment of Cells

The 21MT-2 cells were treated with PMA (100 ng/ml) at 37°C for different times. Total cellular RNA was isolated from cells grown on P100 tissue plates (Falcon, Lincoln Park, NJ) to about 75% confluence. For CAT assays, 21MT-2 cells were transfected with reporter plasmids and cultured for 24 hr before PMA treatment. After PMA treatment, cells were washed with PBS, fresh growth medium was added, and cells were incubated at 37°C for 9 or 30 hr prior to CAT assays.

Results

Identification of SPR1 *by Differential Display*

Total RNAs from 76N and 70N normal breast epithelial cell strains and 21NT, 21PT, 21MT-2, and 21MT-1 breast tumor cell lines were compared by DD. When primer pair OPA1/T_{12}MG was used, a cDNA of 160 base pairs (bp) appeared as a strong band in the 76N lane, was weaker in 70N, 21NT, 21PT, and 21MT-2, but was absent in the 21MT-1 highly invasive tumor cell line (Fig. 1A). This band was excised from the 76N lane, amplified by PCR, and the resulting 160 bp PCR product was ^{32}P-labeled and hybridized to a Northern blot (Fig. 1B). A transcript of 0.6 kb was detected, which was highly expressed in 76N, somewhat lower in 70N, was still lower in 21NT, 21PT, and 21MT-2, and was completely absent in the highly invasive 21MT-1 breast tumor cell line. When the partial cDNA obtained from DD was cloned and sequenced, it was found to be identical to the cDNA encoded by the *SPR1* gene (9,10).

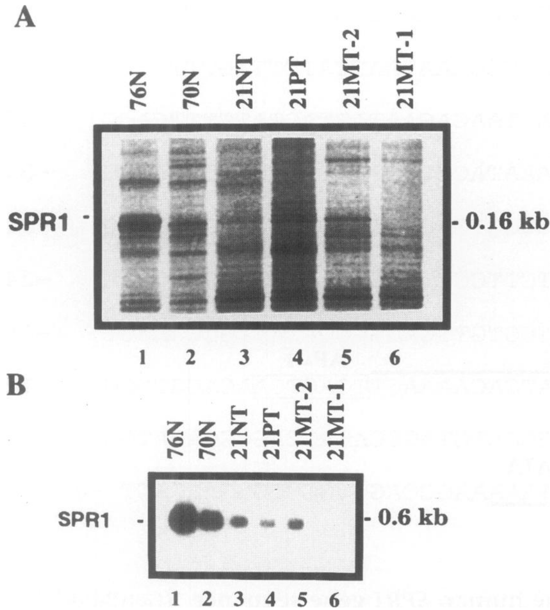

Fig. 1. Identification of the *SPR1* gene by differential display on the basis of its down-regulated or absent expression in metastatic tumor cells. (A) Total RNAs from normal human mammary epithelial cell strains (76N, 70N) (lanes 1,2), primary (21NT, 21PT) (lanes 3,4), and metastatic breast tumor cell lines (21MT-2, 21MT-1) (lanes 5,6) were compared. The resultant cDNAs were resolved by electrophoresis on a 6% acrylamide/urea sequencing gel. The position of the *SPR1* cDNA (~0.16 kb) is marked. (B) Confirmation of *SPR1* differential expression pattern by Northern blotting. Total RNAs from normal mammary epithelial cell strains (76N, 70N) (lanes 1,2), primary (21NT, 21PT) (lanes 3,4), and metastatic mammary epithelial tumor cell lines (21MT-2, 21MT-1) (lanes 5,6) were compared. Each lane contained 10 μg of total cellular RNA. Staining with ethidium bromide showed similar loading of non-degraded RNA in each lane (not shown). The size of the transcript was estimated from molecular size markers run on a parallel lane (not shown).

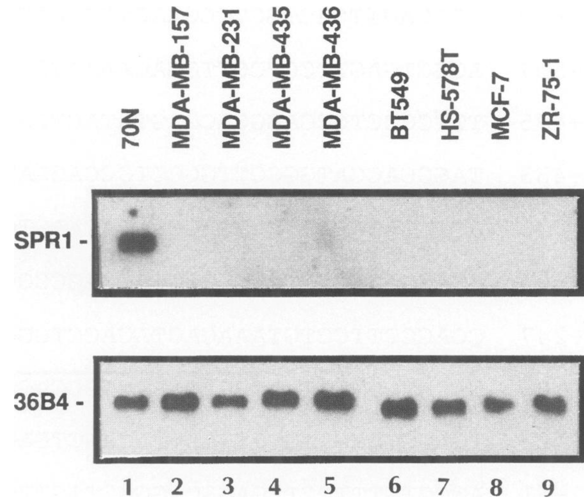

Fig. 2. Northern analysis of *SPR1* gene expression in normal (70N) and tumor human mammary epithelial cell lines (all others) (top). Each lane contains 20 μg of total RNA. The blot was hybridized against a ^{32}P-labeled full-length *SPR1* cDNA probe. Then the blot was stripped and rehybridized to the ribosomal gene 36B4 (22) as an internal loading and transfer control (bottom). The size of the transcript was estimated from molecular size markers run on a parallel lane (not shown).

Expression of SPR1 *mRNA in Normal and Tumor Mammary Epithelial Cells*

The expression of *SPR1* mRNA in normal and tumor mammary cell lines was analyzed by the Northern blot shown in Figure 2. Normal mammary epithelial cell strains such as 76N, as well as 70N and 81N (not shown), express the 0.6 kb *SPR1* transcript. One primary tumor cell line (HS-578T) as well as seven metastatic mammary tumor cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-435, MDA-MB-436, BT549, MCF-7, and ZR-75-1) all failed to express the *SPR1* transcript.

Cloning of SPR1 *Promoter, Generation of CAT Constructs, and Activity of* SPR1 *Promoter in Normal and Tumor Breast Cells*

To analyze the transcription of the *SPR1* gene, its promoter was cloned upstream of the CAT reporter gene. Since the *SPR1* promoter sequence was known (16), it could be obtained by PCR from a genomic clone isolated from a human leukocyte library, or directly from genomic DNA isolated from 70N cells. The sequence of the PCR product obtained from the leukocyte library displayed a total of 13 discrepancies with the published sequence (16). The sequence was confirmed by sequencing a PCR product generated from 70N genomic DNA. The *SPR1* promoter sequence obtained with these two different templates matched exactly and is shown in Figure 3. Subsequently, pBLCAT2 and pBLCAT3 expression plasmids were used to test the activity of the *SPR1* promoter. Deletion mutants were prepared to identify promoter regulatory sequences. The pBLCAT2 plasmid contains the minimal herpes virus TK promoter, which has low basal activity in the absence of additional positive regulatory elements, whereas pBLCAT3 contains neither a promoter nor an enhancer (30). A CAT construct was prepared, which contained the promoter se-

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-619   CCAGAGTTCACTGCTCCGAACTCTCCTTCACAGACTGAGGTCAGCCTGCCCCTATGTTGTTT   -558
-557   ACCCTGAGTTCCTCCTTGGAGAAAAGGCTTTTTAGAACCGAAATAATCATTTTAGTTTTTCC   -496
-495   TTTCCCCTGCCAGGCACGAGTATACTGAGTGATGGATTAACAGAATATTACTATTTTTCACC   -434
-433   TAGCCACCATGGCCTTGGCCTCCCAGAATAACCAGAAATACCTATTGTTTAGCTCTACATCC   -372
-371   ACTTCATATATTAAACAGCCCTACAAGTGTCACCTGTGCTGAGGATTAGACTCTTCCAGAAG   -310
-309   ATAGGACAGTTTCTGGTTCCAGCAGCCCCAGAATGTCTTCCTTCTCTCTCTTTCAGCCCACA   -248
-247   CCACCCTTCCTGTAAACACTACACCTGGGAGCAAAGGGTGTTCAGGGGGATAAAGCCCAGGT   -186
                                                      AP-1
-185   GACATCCTTGTCAGACAGCAAGTGCCACAAGTTTCATCACAAAAGTTGAGTCAACAGGTGGG   -124
-123   TGAGGGAAGAGGGGTGAATCACATCTGACAGGTAAGGAATGTAGGCACAGCAGCCCAGATGG   -62
                                       TATA
 -61   ATCCTGTTTCCTTGAGGCAGGGCTTGTTCCATGCATAAAAAGCCAGTTGGCTGGGAACACT -1
   1   ACCACCAGTTCTAAG  15
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Fig. 3. Nucleotide sequence of the promoter region of the human *SPR1* gene sequence. (GenBank™/EMBL accession number: AF079516; previously published sequence: M84757). The putative transcription start site is numbered 1. The TATA box is underlined. The AP-1 transcription factor binding site is boxed. The sequence −178 to −139, where the repressor was localized, is indicated by a boldface line.

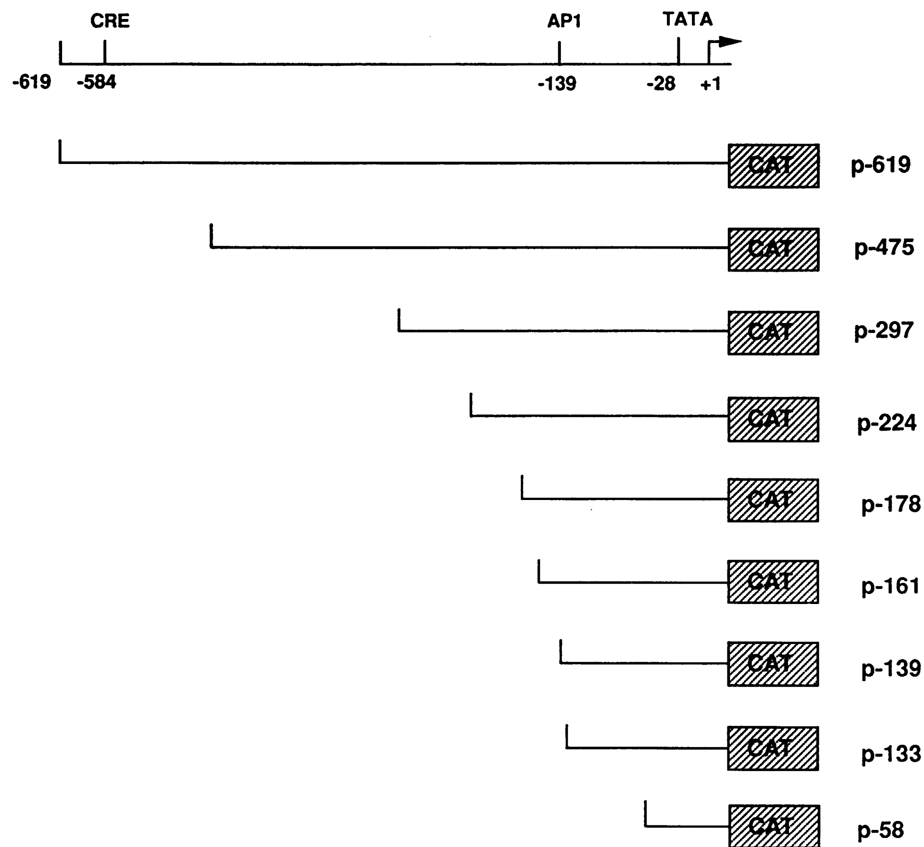

Fig. 4. Schematic representation of the human *SPR1* promoter with putative transcription factor binding sites (top). CAT constructs containing the −619 to +15 of the *SPR1* promoter or 5′-deletions were inserted into the promoterless reporter vector pKT. The putative transcription start site is numbered +1. Numbers corresponding to the 5′-end of the TATA box, the AP-1 binding site, and a putative cAMP-responsive element (CRE) in the *SPR1* 5′-flanking region are shown (top). The location of the 5′-end of each construct in the human *SPR1* promoter is indicated in numbers (bottom).

quence generated from the leukocyte library. For promoter analysis, 5′ deletion constructs (−619, −475, −297, −224, −178, −161, −139, −133, and −58) were generated by cloning the corresponding promoter fragments upstream of the CAT reporter gene (Fig. 4).

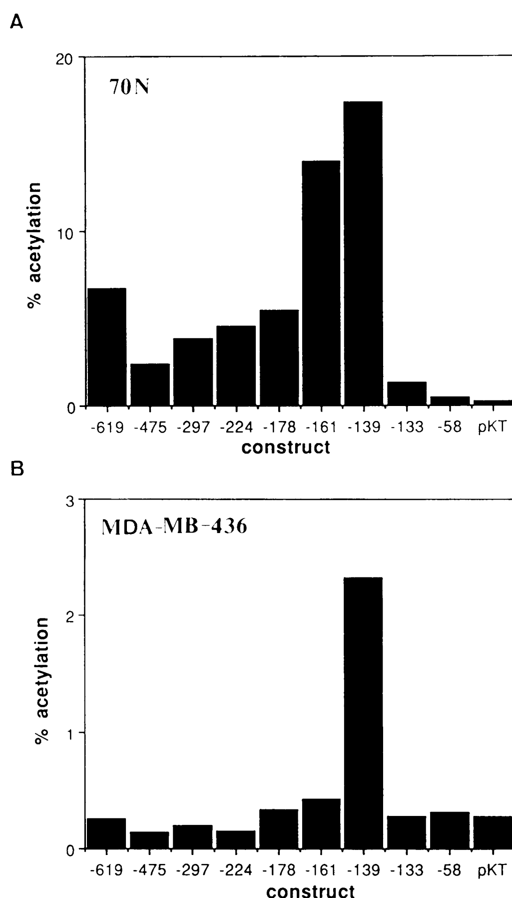

Fig. 5. Deletion analysis of the *SPR1* promoter. pKTCAT constructs were transfected into 70N normal breast cells (A) and MDA-MB-436 breast tumor cells (B). The results were normalized for differences in transfection efficiencies among different cell lines by co-transfecting a β-Gal expression vector and normalizing for β-Gal activity. CAT assays were performed on amounts of extracts giving equal activity. Results are typical of triplicate experiments.

To determine whether the promoter fragment (−619 to +15) is sufficient for transactivation in cells where the endogenous message is produced and whether it lacks activity in cells where the endogenous message is absent, the sequence −619 to +15 of the *SPR1* promoter was inserted into the promoterless CAT vector pKT (p-619) and transfected into various normal and tumor cell lines. The result of a typical experiment is shown in Figure 5A. The 70N cells, which express a high amount of *SPR1* mRNA, produced 5% acetylation, whereas 21NT and 21MT-2 cells, which express lower *SPR1* mRNA levels, produced 1% and 1.5% acetylation, respectively (data not shown). However, tumor cell lines MDA-MB-436 (Fig. 6B), and MDA-MB-231, MDA-MD-435 (data not shown), which express no endogenous mRNA, produced activities comparable to the control vector. Therefore, it can be concluded that the −619 to +15 promoter fragment is sufficient for *SPR1* transactivation in cells that produce some amount of *SPR1* mRNA, but lacks activity in cells with no detectable *SPR1* mRNA. Furthermore, these results suggest that the inactivation of *SPR1* expression in tumor cells likely occurs by a transcriptional mechanism.

Determination of Putative Regulatory Elements in the SPR1 *Promoter*

The 5′-deletion constructs shown in Figure 4 were transfected into 70N cells and CAT activity was determined. Results of a typical experiment are shown in Figure 5A. Transfection of 70N cells with the p-619, p-475, p-297, p-224, and p-178 CAT constructs resulted in acetylation levels ranging from 2% to 7%, significantly higher than the activity of the promoterless construct. The minor variations initially seen between these constructs were not reproduced when the experiment was repeated twice (data not shown). Transfection of the p-161 and p-139 constructs, however, resulted in markedly higher CAT activity, with acetylation levels of 14% and 18%, respectively. However, low acetylation of 1% was observed upon transfection of the p-133 construct, while transfection of the p-58 construct resulted in 0.3% acetylation. The increase in activity observed with the p-161 and p-139 constructs, compared to the p-178 construct, suggests the presence of an inhibitory element located between −178 and −139. The sharp drop in activity between the p-139 and p-133 constructs suggests the presence of a positive enhancer element located between −139 and −133. Our data presented below suggest that the enhancer element, which is required for high levels of *SPR1* transcription, is very likely the AP-1 binding site (TGAGTCA), which lies between −139 and −133.

Are These Regulatory Elements Involved in Down-regulation of SPR1 *Expression in Tumor Cells?*

To determine whether the identified inhibitory element located between −178 and −139 and the positive enhancer element located between −139 and −133 are involved in the down-regulation of *SPR1* expression in tumor cells, the 5′ deletion constructs were transfected into the MDA-MB-436 breast tumor cell line. As seen in Figure 5B, transfection of MDA-MB-436 with

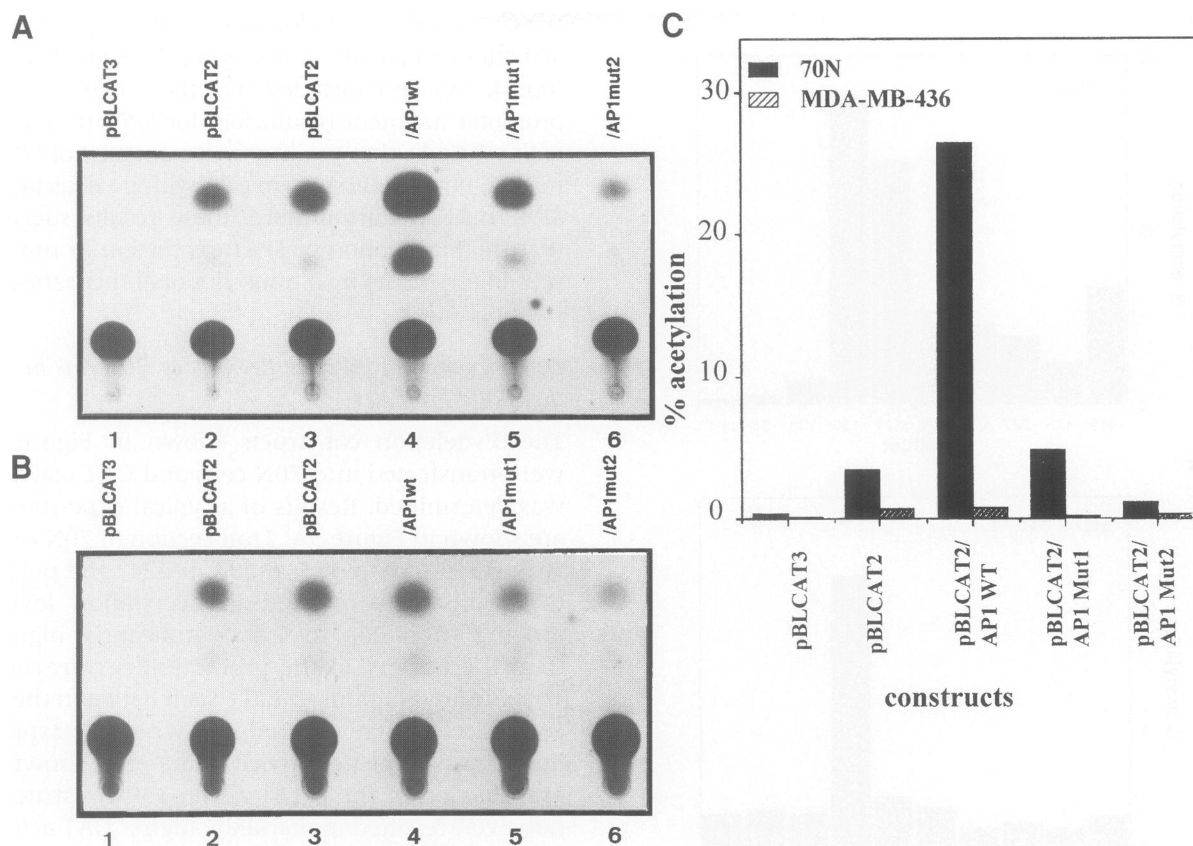

Fig. 6. An intact AP-1 site is required for enhancement of *SPR1* promoter activity. CAT assays were performed in 70N normal breast cells (A) and MDA-MB-436 breast tumor cells (B) and the percentage of conversion of [^{14}C]chloramphenicol to the acetylated form was measured. Values obtained from counting spots in a scintillation counter are shown (C). CAT constructs were transfected containing wild-type AP-1 (lane 4), and mutant AP-1 with two nucleotide changes (lane 5), or with changes in all seven nucleotides of AP-1 (lane 6). The pBLCAT3 promoterless vector and the pBLCAT2 vector with the TK promoter were transfected as negative controls (lanes 1–3). Extracts of 15 β-Gal units were assayed for CAT activity. Results are typical of triplicate experiments.

the p-619, p-475, p-297, p-224, p-178, and p-161 constructs gave acetylation values of 0.1% to 0.35%, similar to the promoterless vector control. However, when the p-139 construct was transfected into MDA-MB-436 tumor cells, acetylation of 2.3% was observed, which suggests that an inhibitory element is located between −178 and −139, in the same region as that found for 70N cells. When MDA-MB-436 tumor cells were transfected with the p-133 or p-58 constructs, 0.2% acetylation was observed again. These results suggest that the AP-1 regulatory element located between −139 and −133 acts as a weak enhancer of transactivation in MDA-MB-436 breast tumor cells, although it was shown to act as a much stronger enhancer in 70N normal mammary epithelial cells. Similarly, AP-1 has a weak enhancing activity in ZR-75-1 and MCF-7 breast tumor lines (data not shown).

AP-1 Element Acts as an Enhancer in Normal Cells but Not in Tumor Cells

Subsequently, the hypothesis was tested that the AP-1 binding site acts as an enhancer in normal cells but not in tumor cells. Basal AP-1-dependent transactivating activity was measured by cloning oligonucleotides containing the wild-type AP-1 sequence of *SPR1* upstream of the enhancerless TK promoter in the pBLCAT2 vector. Similarly, two mutant AP-1, with either two nucleotide changes (AP1 mut1), or all seven changes in the AP-1 site (AP1 mut2) were cloned. The promoterless pBLCAT3 construct produced no activity upon transfection in 70N cells, whereas pBLCAT2 with CAT driven by the TK promoter produced 2.5% acetylation (Fig. 6A,C). However, the construct containing a wild-type AP-1 site upstream of the TK promoter yielded a large stimulation of CAT

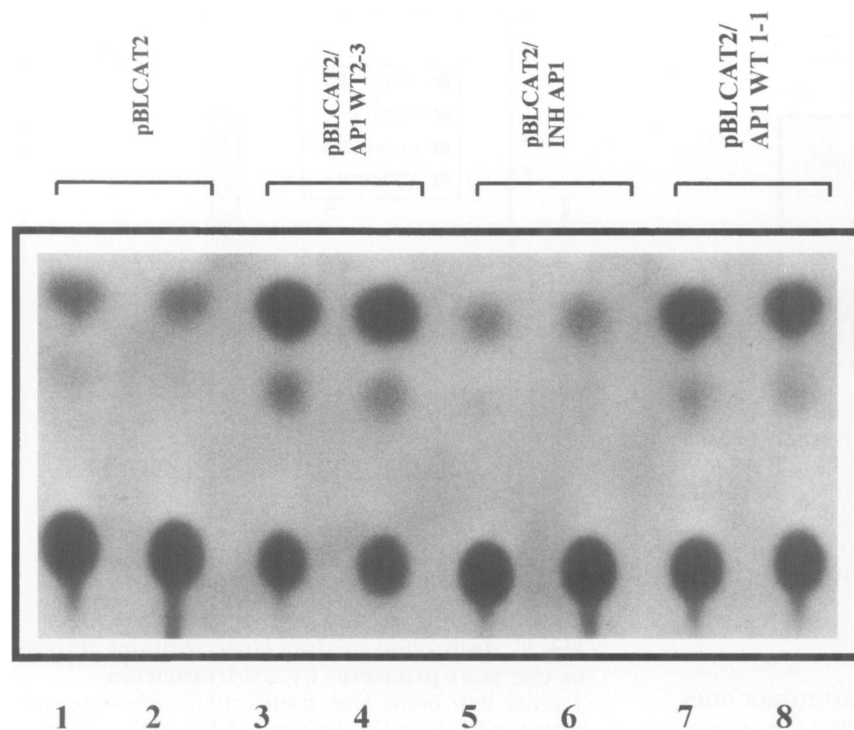

Fig. 7. Effect of AP-1 and the *SPR1* inhibitory element on transcriptional activity. CAT constructs with wild-type AP-1 and altered sequences flanking the AP-1. CAT assays were performed in 70N normal breast cells transfected with pBLCAT2 vector (lanes 1,2); an oligonucleotide containing a wild-type AP-1 site with flanking sequences completely different from those in the SPR1 promoter (pBLCAT2/AP1 WT2-3; lanes 3,4); the *SPR1* wild-type AP-1 site and the inhibitory element (pBLCAT2/INH AP1; lanes 5,6); and the SPR1 wild-type AP-1 site (pBLCAT2/AP1 WT1-1; lanes 7,8). Extracts of 15 β-Gal units were assayed for CAT activity. Results are typical of duplicate experiments.

activity (27% acetylation). This stimulation was not seen with the constructs containing mutations in the AP-1 site. The pBLCAT2/AP-1 mut1 construct, with two mutations, gave 3% acetylation, whereas the pBLCAT2/AP-1 mut2 construct, with all seven positions in AP-1 mutated, gave only 1% acetylation (Fig. 6C). To test whether the sequences flanking the AP-1 site play a putative role in the stimulation of CAT activity, an oligonucleotide containing the wild-type AP-1 site with completely different flanking sequences was transfected into 70N cells. This construct (pBLCAT2/AP-1 WT2-3) produced an 8-fold stimulation of CAT activity compared to pBLCAT2 (Fig. 7, lanes 3,4), which is comparable to the 10-fold stimulation obtained with the oligonucleotide containing wild-type AP-1 and flanking sequences as in the *SPR1* gene (pBLCAT2/AP1 WT1-1) (Fig. 7, lanes 7,8). Therefore, it was concluded that the wild-type AP-1 site acts as a strong enhancer in 70N cells. Furthermore, the flanking sequences surrounding the AP-1 sequence are of minor importance in mediating this enhancing effect. Moreover, in normal 70N cells, the inhibitory element (−178 to −139) can suppress the transcriptional activity of a wild-type AP-1 site, as in the construct pBLCAT2/INH AP-1 (Fig. 7, lanes 5,6), which contains the inhibitor upstream of AP-1, like the *SPR1* promoter.

We sought to determine whether the AP-1 site can act similarly in tumor cells by transfecting the same constructs into MDA-MB-436 tumor cells in which CAT activity was determined (Fig. 6B). The pBLCAT3 construct produced only 0.1% acetylation, while pBLCAT2 produced 0.5%. Similarly, the construct containing wild-type AP-1 resulted in no increase in CAT activity (0.5% acetylation) and constructs having mutant AP-1 sites resulted in further reduction of CAT activity (0.1% acetylation) (Fig. 6C). Therefore, in MDA-MB-436 cells the AP-1 site of *SPR1* does not act as an enhancer for the heterologous TK promoter.

Up-regulation of SPR1 *Expression in 21MT-2 Tumor Cells by UV Irradiation*

Since the *SPR1* mRNA was originally identified as being stimulated by short-UV light in keratinocytes (9,10), we investigated whether UV irradiation could likewise stimulate expression of the *SPR1* message in breast tumor cells. When 21MT-2 cells were irradiated with 25 J/M^2 short-UV light, a reproducible induction of *SPR1* mRNA was observed, which reached a maximum at 6 hr post-UV treatment (data not shown). Figure 8 shows the result of a dose–response experiment. The maximum stimulation of *SPR1* expression was observed with a dose of

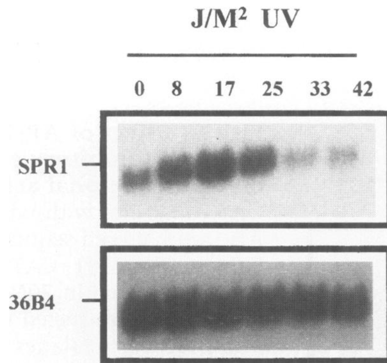

Fig. 8. Northern blot analysis of *SPR1* induction by UV irradiation of 21MT-2 human breast tumor cells. Cells were irradiated with increasing doses of short-UV light. Each lane contains 20 μg of total RNA. The blot was hybridized with a 0.6-kb *SPR1* cDNA probe (top) and 36B4 (22) was used as an internal loading and transfer control (bottom).

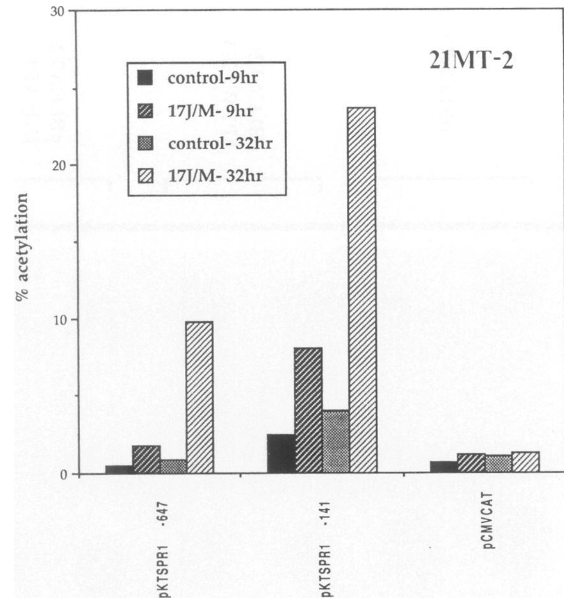

Fig. 9. Induction of the transcriptional activity of the *SPR1* promoter by UV irradiation. Twenty-four hours after transfection, cells were irradiated with short-UV light at 17J/M^2. CAT activity was measured 9 and 32 hr after UV irradiation. Extracts were normalized for β-Gal activity. Values for both irradiated cells and non-irradiated controls are typical of triplicate experiments.

17J/M^2. However, when other breast tumor lines (ZR-75-1, MDA-MB-436, and MCF-7) were examined 6 hr after UV treatment with 17 or 35 J/M^2, no *SPR1* message could be detected in either case (data not shown). These results suggest that transcriptional activity can be stimulated by UV to up-regulate *SPR1* in cells that produce some amount of *SPR1* message. Cells that produce no detectable *SPR1* message probably have a blockage in *SPR1* gene expression that cannot be overriden by UV treatment.

Up-regulation of SPR1 *Expression by UV Treatment Is Mediated by a Transcriptional Mechanism*

To elucidate whether UV treatment up-regulates *SPR1* expression by a transcriptional mechanism, 21MT-2 cells were transfected with either of two *SPR1* promoter–driven CAT constructs (pKT*SPR1*-647 or pKT*SPR1*-141) or with pCMV-CAT, and 16 hr later they were UV-treated at a dose of 17J/M^2. CAT assays were performed 9 or 32 hr after UV irradiation. When the pKT*SPR1*-647 plasmid was transfected, a small increase in the transcriptional activity was observed after 9 hr, which became more prominent after 32 hr, with acetylation levels of 10% (Fig. 9, left). Transfection of the pKT*SPR1*-141 construct and hence, deletion of the inhibitory element resulted in much higher increase in CAT activity, with acetylation levels of 8% and 23% after 9 and 32 hr, respectively (Fig. 9, middle). This result confirms the presence of an active inhibitor regulating the activity of *SPR1* promoter in both normal and tumor cells. On the other side, transfection of the pCMVCAT control construct containing the strong CMV promoter gave no substantial increase in CAT activity under the same conditions (Fig. 9, right). This result shows that the transcriptional activation observed after UV irradiation is specifically dependent on the pKT*SPR1* promoter.

Re-expression of SPR1 *in 21MT-2 Breast Carcinomas by PMA*

Given the presence of an AP-1 consensus site in the *SPR1* promoter, we tested whether activation of the PKC signal transduction pathway by PMA would activate the re-expression of *SPR1* in 21MT-2 tumor cells. The induction of *SPR1* mRNA 3 hr after PMA treatment is shown by the Northern blot in Figure 10 and was detected over a period of 3–24 hr. The increase in *SPR1* mRNA levels was determined from Northern blots by densitometry to be about 10-fold after 6 hr and more than 40-fold after 24 hr of PMA treatment. Thus, up-regulation of *SPR1* expression by PMA was more prominent than its induction by short-UV irradiation. Furthermore, up-regula-

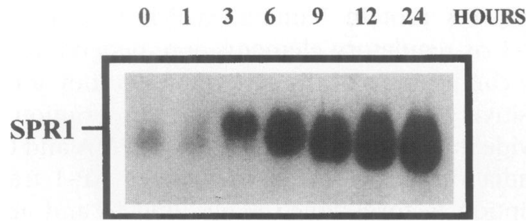

Fig. 10. Northern blot analysis of *SPR1* induction by PMA treatment of 21MT-2 human breast tumor cells. Each lane contains 20 μg of total cellular RNA. The PMA concentration used was 100 ng/ml. The blot was hybridized with a 0.6-kb *SPR1* cDNA probe. 36B4 (22) was used as an internal loading and transfer control (not shown).

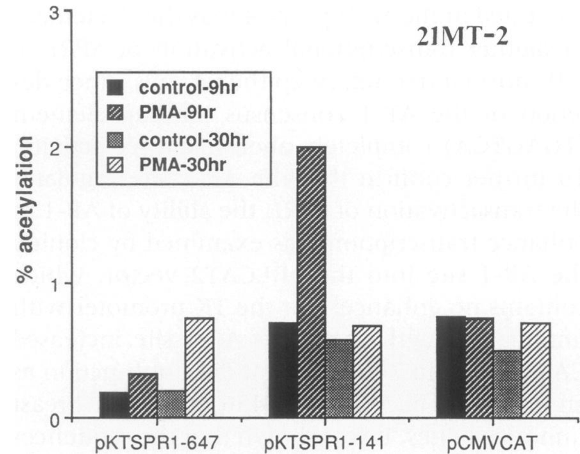

Fig. 11. Stimulation of transcriptional activity of *SPR1* promoter by PMA. Twenty-four hours after transfection, 21MT-2 cells were treated with PMA or without PMA (control) for 9 or 30 hr. PMA was then removed and CAT activity was measured. Extracts were normalized for β-Gal activity. Results are typical of triplicate experiments.

tion of *SPR1* expression by PMA is primarily transcriptional and is not due to increased stability of the *SPR1* mRNA, since RNA stability studies showed a $t_{1/2}$ of about 12 hr for the *SPR1* message in both 76N normal and 21MT-2 tumor cells (data not shown).

Induction of SPR1 *Expression by PMA Is Mediated by AP-1 site*

To elucidate whether PMA up-regulates *SPR1* by a transcriptional mechanism, 21MT-2 cells were transfected with either of two *SPR1* promoter–driven CAT constructs or with pCMVCAT and 16 hr later they were stimulated with PMA. Stimulation of *SPR1* expression was confirmed in each experiment by parallel Northern blot analysis (data not shown). CAT assays were performed 9 or 30 hr after PMA treatment (Fig. 11). When the pKT*SPR1*-647 plasmid was transfected, a small increase in the transcriptional activity was observed after 9 hr, which became more prominent after 30 hr (Fig. 11, left). Deletion of the inhibitory element resulted in a higher increase in CAT activity after 9 hr of PMA treatment observed upon transfection of the pKT*SPR1*-141 construct (Fig. 11, middle), a result that confirms the presence of an active inhibitor in the *SPR1* promoter. Finally, transfection of the pCMVCAT control construct gave no substantial increase in CAT activity under the same conditions (Fig. 11, right), showing that the transcriptional activation of *SPR1* is specifically dependent on the pKT*SPR1* promoter.

Discussion

Differential display was applied to the isolation of transcriptionally regulated genes involved in mammary carcinogenesis. A known gene, *SPR1*, was identified as being down-regulated or inactivated in human breast epithelial tumor cell lines. Expression of *SPR1* in normal and tumor-derived cells is differentially regulated at the transcriptional level. The mechanism of down-regulation of *SPR1* expression in tumor cells has been studied by analyzing the *SPR1* promoter. The promoter of the *SPR1* gene was cloned from a human lymphocyte genomic DNA library, and was also amplified by PCR of a genomic DNA template isolated from 70N normal breast cells. The promoter fragment −619 to +15 was sufficient for activation of *SPR1* transcription in normal mammary epithelial cells, however, it was transcriptionally silent in most breast tumor cells, indicating that the reduced or absent expression of *SPR1* in breast tumor cells is likely due to its transcriptional down-regulation. In agreement with a mechanism of transcriptional down-regulation of the *SPR1* gene in breast tumor cells, Southern blot analysis showed uniform patterns of restriction fragments in both normal and tumor cell lines, suggesting that the *SPR1* gene contains no gross structural alterations in breast tumor cell lines, although point mutations cannot be excluded (data not shown). To identify the functional *SPR1* promoter elements, 5′ deletion mutants were transiently transfected into 70N normal breast cells and several breast tumor cell lines and their relative CAT activity was assayed. An AP-1 binding site was

identified in the *SPR1* promoter as the *cis* element mediating transcriptional activation of *SPR1* in 70N normal mammary epithelial cells, since deletion of the AP-1 consensus binding element (TGAGTCA) completely abolished CAT activity. To further confirm that the AP-1 site regulates the transactivation of *SPR1*, the ability of AP-1 to enhance transcription was examined by cloning the AP-1 site into the pBLCAT2 vector, which contains no enhancer but the TK promoter with minimal strength (24). This AP-1 site increased CAT activity in 70N cells, but did not function as an enhancer in MDA-MB-436 and other breast tumor cell lines. It was shown that the sequences flanking the AP-1 site do not affect its promoter-enhancing activity. In addition, a major upstream negative regulatory element is located between −178 and −139 in the *SPR1* promoter, which is active in both normal and tumor cells. It should be noted that an E-26 transformation-specific site (Ets) is present at −55 of the *SPR1* promoter sequence; however, it gives low CAT activity if AP-1 is deleted (data not shown). Our results demonstrate that down-regulation of *SPR1* expression in breast tumor cells results from lack of transactivation through AP-1 in combination with a modulated negative regulation through an inhibitory element located between −178 and −138, which appears more active in tumor cells. This mechanism of aberrant gene expression might be common in tumor cells. For example, transcription of *maspin*, a tumor-suppressing serpin, is coordinately regulated in prostate cells by a hormone-responsive negative element (HRE) recognized by the androgen receptor that is active in both normal and tumor cells and by an activating Ets binding site, which is inactive in tumor cells, thus, resulting in reduced or absent expression of maspin in prostate tumor cells (31). In breast cells, loss of maspin expression during tumor progression results from the absence of transactivation through the Ets and AP-1 sites (32).

It was shown that loss of *SPR1* expression can be restored in breast tumor cells by PMA treatment and by UV irradiation. However, expression of *SPR1* could be stimulated by PMA and UV irradiation only in breast tumor cell lines in which *SPR1* is down-regulated, e.g., 21MT-2, but not in tumor cell lines in which the *SPR1* message is totally absent, e.g., MCF7. PMA is an activator of the PKC signal transduction pathway (33). The PKC pathway initiates a phosphorylation cascade that ultimately activates the expression of AP-1 transcription factors, namely *c-Jun* and *c-Fos* protein families, which bind to the AP-1 *cis*-regulatory elements and, depending on the composition of the heterodimers they form, positively or negatively regulate transcription of a wide number of target genes (34). PMA and UV irradiation induce the expression of AP-1 transcription factors, which likely bind to and activate transcription through the AP-1 site in the *SPR1* promoter. Induction of AP-1 factors by PMA in 21MT-2 cells was previously shown by our group (35). A rapid increase in *c-Jun* and *c-Fos* mRNA levels was observed 1 hr after PMA treatment of 21MT-2 cells, whereas *JunD* was not induced (35). Untreated 21MT-2 contained little AP-1 binding factor. This transcriptional activation is regulated by phosphorylation of *c-Jun* and *c-Fos* proteins at specific sites (36). PMA treatment of 21MT-2 possibly triggers the kinase involved in phosphorylation of AP-1 factors. For example, PMA induction of the *elafin* gene in 21MT-2 not only involves induction of *c-Fos* and *c-Jun* but also depends on phosporylations that activate AP-1 factors through the PKC signal transduction pathway (35).

Previous studies of keratinocyte transformation (37) have demonstrated that AP-1 activity increases as keratinocytes become transformed by 12-o-tetradecanoyl-phorbol-13-acetate (TPA) or epidermal growth factor (EGF) stimulation, and that blockage of AP-1 prevents transformation. Similarly, an increase in AP-1 activity was observed when mouse epidermal cells were transformed by X-irradiation (36), while AP-1 is shown to be involved in tumor promoter-induced transformation (38). In contrast, our results support recent studies suggesting that normal human mammary epithelial cells have higher AP-1 DNA binding and transactivating activities than human breast cancer cells, with a progressive decline in AP-1 transactivating activity as cells progress through the carcinogenesis pathway (39). In summary, higher AP-1 activity has been reported for some tumors (36–38,40), whereas lower AP-1 activity was determined in others (35,39). Therefore, common transcription factors such as AP-1 are activated by tissue-specific signaling pathways and transformation likely occurs via distinct molecular pathways in different cell types. The differences in AP-1 activity in normal and malignant cells may indicate that growth of normal mammary epithelial cells is more dependent on signals mediated by AP-1 than mammary tumor cells. The reduction of AP-1 activity that occurs

during transformation of human mammary epithelial cells may also be occurring via other signal transduction pathways only active in tumor cells.

UV radiation activates the expression of a wide variety of genes. Short-wavelength UV, like other extracellular stimuli including growth factors, activates signal transduction events that involve both stress- and mitogen-activated protein kinase cascades (41). Although both *SPR1* and *SPR2* proteins were described as being induced by UV, and a CAT activity enhanced by UV was demonstrated in transfected cells, no UV-responsive consensus elements could be identified in the 5′-flanking region of *SPR1* or the three *SPR2* genes (42). Since UV quickly activates the expression of c-*Fos* and c-*Jun* (43), it possibly acts through the same pathway as PMA, and transactivates the *SPR1* gene expression through the AP-1 binding site in its promoter. This provides a possible explanation for why only those breast tumor cells that produce some amount of *SPR1* message can be stimulated by UV to up-regulate *SPR1* expression. It should be noted that *SPR1* induction by PMA measured by Northern blots is much higher than the activity measured by CAT assays. Induction of *SPR1* by PMA may result from a cooperative transactivation through the AP-1 and putative unidentified upstream or downstream element(s), missing in CAT constructs. Alternatively, the mechanism of stimulation of *SPR1* transcription by PMA could be more complex than its activation induced by UV irradiation, and artificial constructs probably cannot substitute for the native transcriptional machinery.

A major aim of expression genetics in cancer is to specifically correct aberrant gene expression in tumors by the application of therapeutic agents. PMA is toxic; however, induction of *SPR1* in breast tumor cells could similarly be induced by drugs acting on the same signal transduction pathway as PMA. If, indeed, genes are coordinately regulated in a network, change in expression of one gene can result in the re-expression of multiple other genes in a coordinate manner, leading to a substantial normalization of tumor phenotypes with obvious therapeutic implications (1,44).

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